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The fate of full bovine papillomavirus (BPV) virions and virus-like particles after binding to C127 or CV-1 cells was studied by electron microscopy and indirect immunofluorescence. After incubation at 4° for 1 hr, BPV virions were found to be bound to the plasma membrane, and most viruses were absorbed by the cells after 30 min incubation at 37°. Ninety minutes after the virions had been bound to the plasma membrane, the uptake of the virions was completed and most of the antigen was found to be localized in the nucleus. The viruses were transported in phagosomes and the uptake and transportation could be inhibited by cytochalasin B and taxol, suggesting the possible involvement of microfilaments and microtubules in the virus particle uptake and transportation. The capsid proteins could be detected for about 14 hr, until degradation and deposit of the viral antigen in the Golgi complexes. Although binding to the plasma membrane and uptake of virions into large cytoplasmic vesicles could be monitored by electron microscopy, no complete virions were observed in the nucleus of infected cells despite a very strong nuclear fluorescent staining for both L1 and L2 proteins. This may indicate that disintegration of the virions occurs in the cytoplasm and the L1/L2 proteins migrate to the nucleus via their nuclear localization signals.

INTRODUCTION

Papillomaviruses (PV) are epitheliotropic double-stranded DNA viruses. The virions are nonenveloped and have a 55-nm icosahedral structure (Crawford and Crawford, 1963; Galloway and McDougall, 1989), which is comprised of the L1 major and the L2 minor capsid proteins (Favre *et al.,* 1975; Pfister and Fuchs, 1987). The recent high-resolution cryoelectron microscopic analysis of bovine papillomavirus 1 (BPV1) and HPV1 virions has determined that the two viruses have a very similar structure, with 72 pentameric capsomeres, each presumably being composed of five L1 molecules, forming a virion shell with a $T = 7$ symmetry (Baker *et al.,* 1991). The early phase of the papillomavirus infection has remained unclear despite of its obvious importance for understanding papillomavirus pathogenesis, mostly because there is no *in vitro* cell culture system which allows the vegetative reproduction of HPV virions.

Recent studies, by us and others, provided evidence that animal and human papillomaviruses may enter the cell through the same route, presumably via an ubiquitous receptor molecule (Roden *et al.,* 1994a; Volpers *et al.,* 1995; Müller *et al.,* 1995).

Like all other DNA viruses infecting eukaryotic cells, with the exception of the poxviruses (Moss, 1990), the successful infection of PVs depends upon the transfer of the viral genome into the nucleus, where gene transcription, DNA replication, and viral maturation take place. Virions must attach to a receptor on the cell surface, followed by a process of internalization and penetration through the plasma membrane. Uncoating and release of the viral nucleic acid might occur in the cytoplasm or, in case of, e.g., SV40 and polyomavirus, after relocation of the virions to the nucleus (Maul *et al.,* 1978; Hummeler *et al.,* 1995). However, in case of the PV little is known about these processes. We have studied these early events of PV infection by using BPV virions and HPV virus-like particles, and the pathway of PV infection was documented using electron microscopy and immunofluorescence.

MATERIALS AND METHODS

Purification of BPV virions from cattle warts

All steps of purification were carried out at 4° except those otherwise indicated. About 2 kg of cattle wart was minced, mixed with 3 liters of extraction buffer (20 m*M* HEPES, pH 7.4, 10 m*M* $MgCl_2$, 50 m*M* $CaCl_2$, 150 m*M* NaCl, 0.01% Triton X-100, and 1 m*M* PMSF), and homogenized for 15 min using a Polytron (PT 10/35, Brinkmann Instruments) homogenizer. The suspension was then cleared in a Sorval GS3 rotor for 20 min at 6000 rpm. The supernatant was centrifuged in a Sorval AH629 swinging bucket rotor for 2 hr at 27,000 rpm and the pellet resus-

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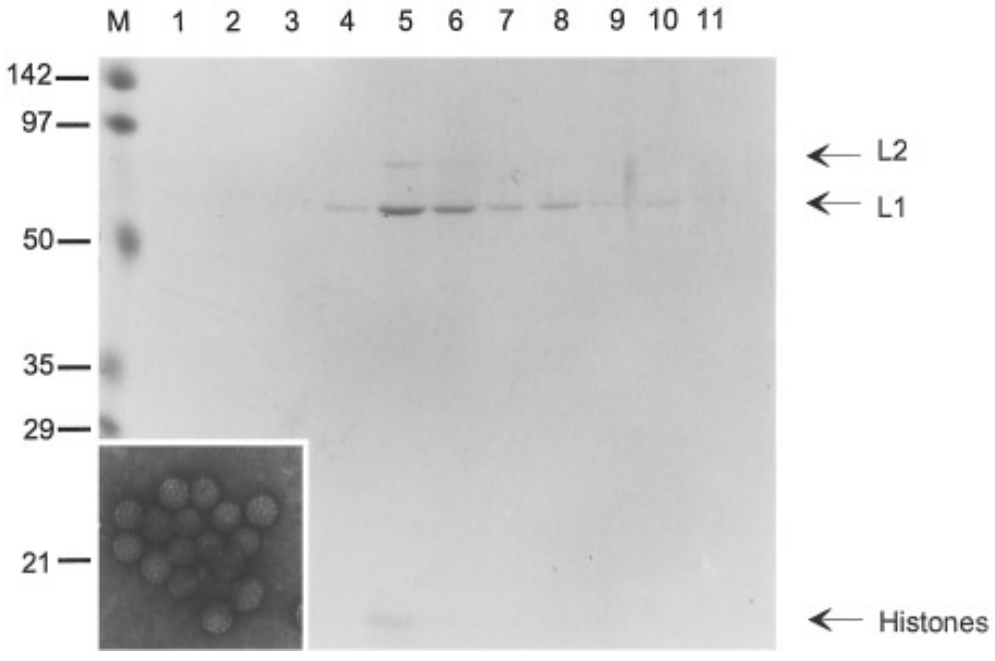


FIG. 1. CsCl purification of BPV virions from a cattle wart. Virions were purified as described under Materials and Methods by CsCl density centrifugation. The gradient was fractionated and 5 μl of each sample loaded onto a 10% SDS–polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue. The insert shows an electronmicrograph of the purified virions.

pended in 50 ml of extraction buffer followed by centrifugation through a 15-ml sucrose cushion (40% sucrose, 50 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, 150 m*M* NaCl, 20 m*M* HEPES, pH 7.4) for 2 hr at 27,000 rpm in the AH629 rotor. The pellet was again resuspended in 20 ml extraction buffer. After addition of 5 g CsCl the suspension was filled into 13.4-ml quickseal tubes and centrifuged for 14 hr in a Beckman 70Ti rotor at 45,000 rpm. The tubes were then punctured and fractionated in 1.5-ml aliquots. For analysis of protein purity 5 μl of each fraction was loaded on a SDS–polyacrylamide gel and stained with Coomassie brilliant blue (see Fig. 1), respectively blotted onto nitrocellulose, and the BPV proteins were detected using a polyclonal rabbit anti-BPV antiserum (Dako). The purified BPV virions were also examined by negative staining and electron microscopy as described in Müller *et al.* (1995) (Fig. 1).

Indirect immunofluorescence

Cells grown on glass coverslips were infected with purified BPV virions, 2 μg per 10^4 cells. This high dose of input BPV virion was required in order to visualize signal from incoming viral proteins. Virus was bound to cells in the presence of 10% FCS at 4° for 1 hr with gentle shaking. The cell monolayers were washed with ice-cold medium three times to remove unbound virions. To allow internalization of virions, monolayers were shifted to 37° and analyzed at various time points. The cells were then washed with PBS, fixed with 80% ethanol for 5 min, and processed for indirect immunofluorescence. Monoclonal antibody (MAb) 5B6 (Roden *et al.,* 1994a) was used at 1:100 dilution for the detection of BPV L1, and a polyclonal rabbit anti-BPV L2 (full length) (Roden *et al.,* 1994b) was applied to identify the L2 protein. (Both antibodies were kindly provided by J. Schiller, NIH.) After washing to remove unbound antibodies, FITC-linked anti-mouse IgG (Sigma) and Texas red-conjugated anti-rabbit IgG (Amersham), both at 1:50 dilution, were used to detect L1 (green) and L2 (red) proteins of BPV virions. For double staining of lysosomal vesicles and BPV L1, rat anti-lysosomal marker lamp-1 (Chen *et al.,* 1985) and rabbit anti-BPV L1 (Dako) antibodies were applied to cells infected with BPV for 30 min followed by anti-rabbit IgG (Texas red-linked) to detect BPV L1 and anti-rat IgG (FITC-linked) to stain for lysosomes. For Golgi complex and BPV co-staining of cells infected with BPV overnight, anti-Golgi β COP MAb (Sigma) and rabbit anti-BPV (Dako) were used to identify Golgi apparatus and BPV. Anti-mouse (Sigma) (FITC-conjugated) and anti-rabbit (Amersham) (Texas red-linked) antibodies were used to identify Golgi (green) and BPV (red) particles.

Electron microscopy

To visualize BPV bound to or already internalized into cells, 2×10^5 CV-1 cells grown on 18-mm cover slides were washed with PBS and about 10 μg of BPV virions was added to the cell monolayers. After incubation at 4° for 1 hr, cells were washed with PBS to remove unbound BPV and fixed for 30 min with 2.5% glutaraldehyde in PBS containing 1 m*M* $MgCl_2$. After fixation with osmium

tetroxide, the cells were dehydrated with an increasing concentration of ethanol and embedded in epoxide resin. Sections were cut and stained with 1% uranyl acetate and lead citrate. The sections were examined with a Zeiss EM 10A microscope.

Chemicals

Chloroquine, butylamine, colcemid, NH_4Cl, and colchicine were dissolved in water. Cytochalasin B, podophyllotoxin, and taxol were dissolved in DMSO. Monensin and vinblastine were dissolved in 50% ethanol. In all experiments, stock solutions (1000×) of the chemicals were added directly to the culture media. The cells were incubated with the above compounds for 2 hr at 37° and washed with fresh medium before BPV virions were added for binding and penetration assays. After the BPV were bound to the cells at 4°, the monolayers were washed with PBS to remove free BPV virions. Medium containing chemicals was added again and the cells were incubated at 37° for different time points.

RESULTS

Purification of BPV virions

Bovine papillomavirus virions were purified from cattle warts as described under Materials and Methods. The quality and quantity of the preparations were controlled by SDS–PAGE and electron microscopy. The viral protein bands for L1 and L2 appearing in fractions 5 and 6 (Fig. 1), corresponding to a gradient density of 1.34–1.35 g/ml, indicate the presence of full virions in these fractions, whereas protein bands in fractions 7 or higher represent empty and therefore lighter capsids. As judged from the gel, the virions were more than 95% pure and consisted of the L1, L2, and histone proteins. By electron microscopy, most of the virions appeared "full," i.e., they seem to contain the nucleic acid (Fig. 1 insert). Similarly, SV40 virions were purified from infected Vero cells and the preparations were analyzed accordingly.

BPV binding and penetration into cells

To study the time course of BPV particle binding, uptake, and transport, subconfluent monolayers of monkey kidney epithelial cells (CV-1) or mouse fibroblasts (C127) were exposed to large amounts of BPV virions (estimated 10^6 virions per cell). No differences in BPV penetration and transportation were noted between the two cell lines. The virions were allowed to bind to the cells for 1 hr at 4°. The cells were then washed and the temperature was shifted to 37°. After different time points the cells were fixed either with 80% ethanol or 4% paraformadehyde and permeabilized with 0.1% saponin (Sigma). No differences were noted with the two fixation methods; ethanol fixation was, therefore, used in indirect immunofluorescence staining. The virions were detected by electron microscopy or by indirect double-label immunofluorescence, which allowed the detection of the L1 and L2 capsid proteins. In the following the time course of BPV binding and penetration is presented (Fig. 2).

At 0 min

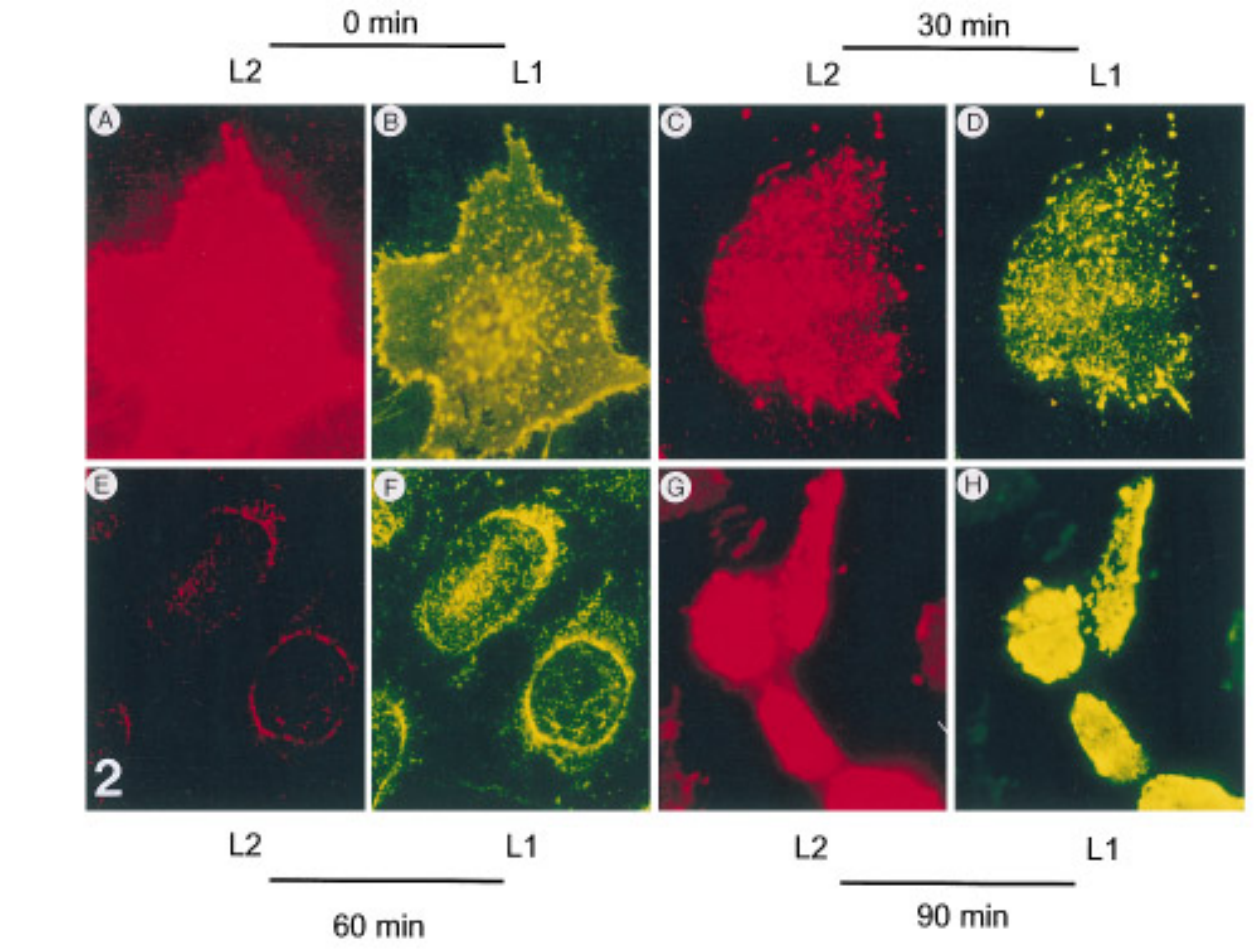
Attachment of BPV at 4° demonstrated that virions were able to attach but not penetrate the cell under low-temperature conditions. The entire surface of the cell was found to be covered with virions. This suggested that the cellular receptor sites are evenly distributed over the cell surface, as opposed to being concentrated on one particular area (Figs. 2A and 2B). Although no polarization of the staining was seen, the signals were located on protrusions of the cell surface in a fiber-like pattern. The dot pattern observed in Fig. 2B resulted from cell surface-bound virions. Using the electron microscope, arrays of full BPV virions could easily be detected on the plasma membrane (some of them in coated pits) as shown in detail in Figs. 4A and 4B. Staining with either anti-L1 or anti-L2 antibodies (compare Figs. 2A and 2B) revealed a strict colocalization of both papillomavirus capsid proteins.

At 30 min

After shifting the temperature for $\frac{1}{2}$ hr to 37° to allow uptake of virions, we observed that a group of virions appears to be attached tightly to the cell surface, and the membrane appeared to be slightly depressed beneath the virus particle (Fig. 4B, see arrow). Penetration of BPV virions across the plasma membrane was observed to occur in most of the cells. The plasma membrane appeared to surround the virions tightly and engulf them into the cytoplasm, pinching them off from the plasma membrane and forming an endocytic vesicle (Fig. 4C). The transport of the virions into the cytoplasm was almost completed after 30 min (Figs. 2C and 2D). While some of the fluorescence signals were still found on the plasma membrane the majority of the virions had obviously been taken up and were now located in the cytoplasm. The signals were concentrated as lumps throughout the whole cell. By electron microscopy, the virions were found to stay in a clustered fashion in large vesicles (400–800 nm in diameter), probably endosomes (see Figs. 4A and 4D). Although the vast majority of the BPV virions were located in uncoated endosomes, occasionally vesicles containing coated pits could be observed. Also, the L1 and the L2 proteins were both colocalized in the cytoplasm, indicating a tight association of the two proteins.

At 60 min

After shifting the temperature to 37°, most of the BPV antigen could be detected in a perinuclear zone (Figs.



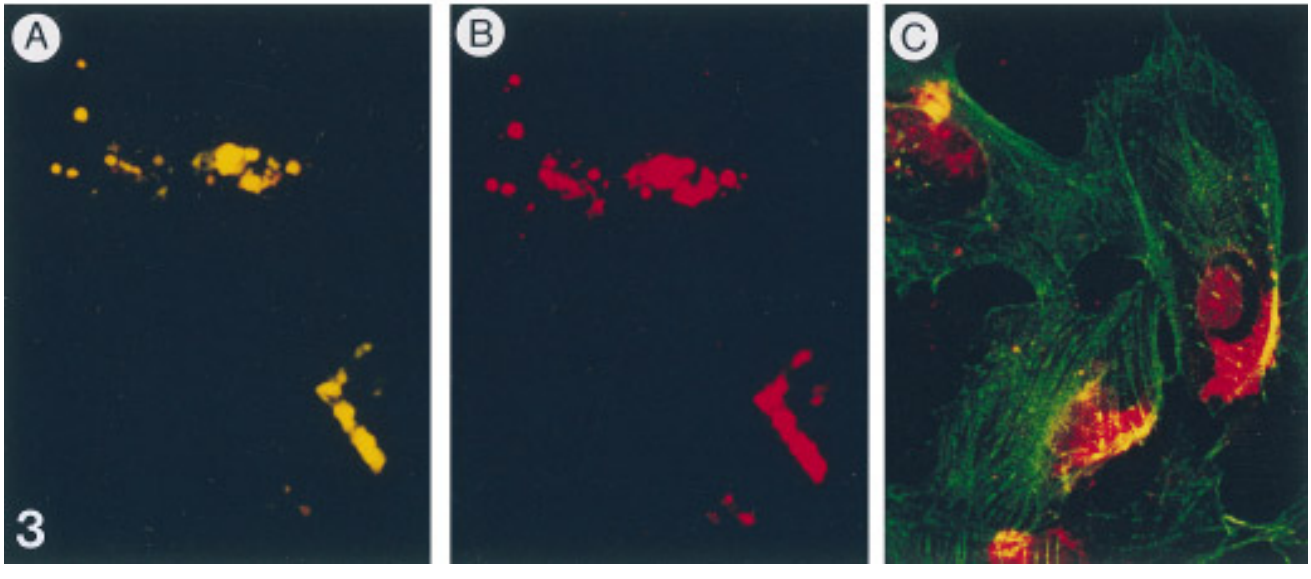
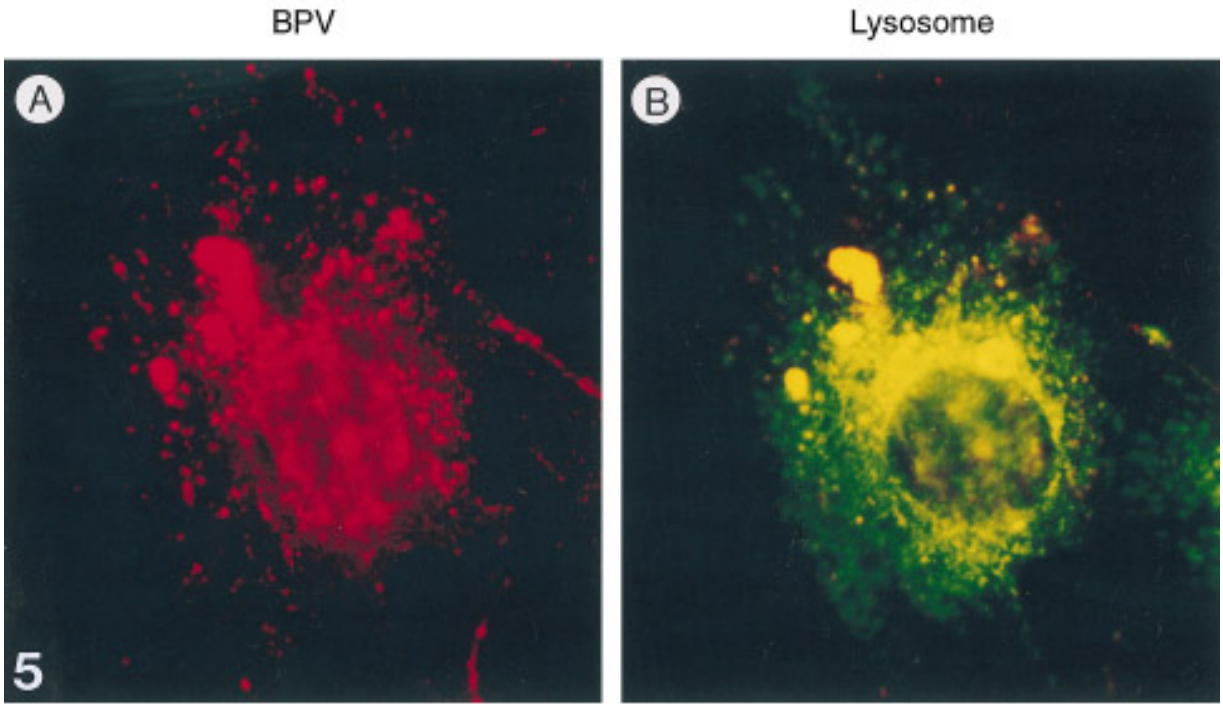


FIG. 2. Time course of BPV infection. BPV virions purified from a cattle wart were bound to CV-1 cells for 1 hr at 4°. The temperature was then shifted to 37° to allow particle uptake. Cells were then fixed at 0 (A, B), 30 (C, D), 60 (E, F), and 90 min (G, H), respectively, and the L1 (B, D, F, H), and the L2 (A, C, E, G) proteins were detected by indirect immunofluorescence. Predominant staining of the cytoplasmic membrane at 0 min, of the cytoplasm after 30 min, of the nuclear membrane after 60 min, and of the nucleus after 90 min was observed. At all time points a colocalization of L1 and L2 is evident.

FIG. 3. Golgi apparatus location of BPV virions 16 hr after particle uptake. Cells were infected with BPV virions for 1 hr at 4° as described under Materials and Methods. The temperature was then shifted to 37° for 16 hr and the cells were subsequently prepared for immunofluorescence. (A) Immunofluorescence staining for the Golgi apparatus specific antigen β COP; (B) immunofluorescence staining of BPV L1; (C) double immunofluorescence staining of BPV L1 and the cellular actinin to demonstrate cellular localization of the BPV antigen. Note that A and B show the same field; C, however, shows a different field.



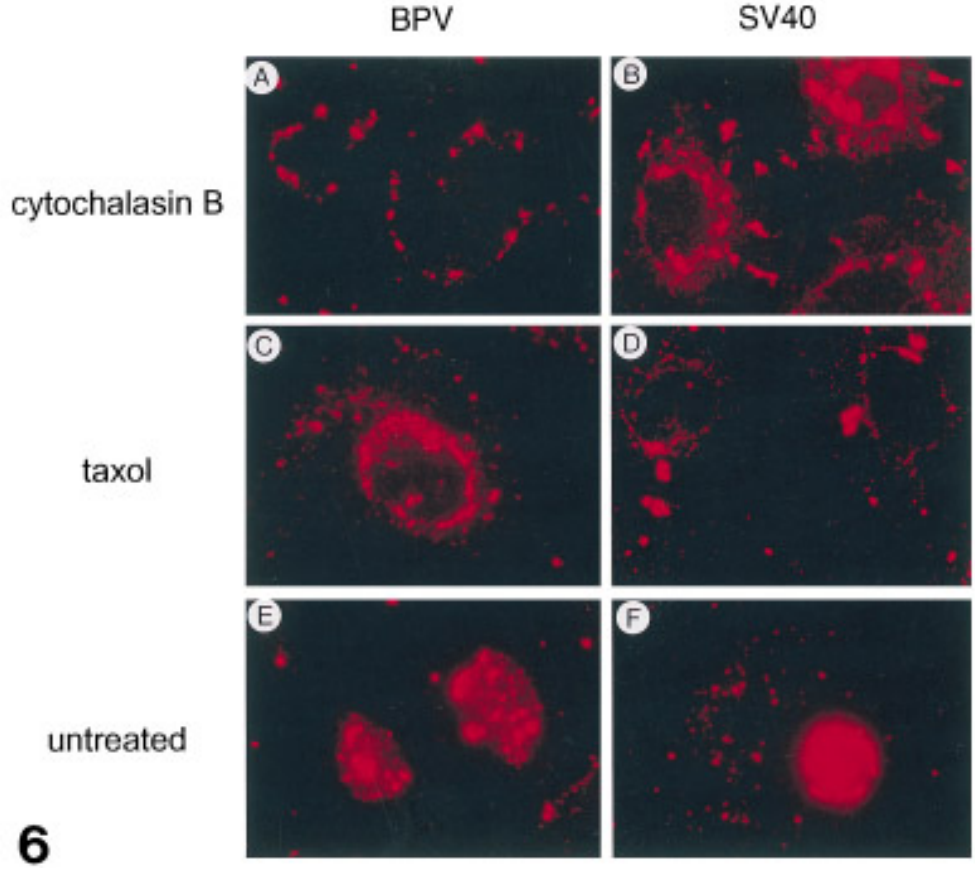


FIG. 5. Colocalization of BPV antigen with the lysosome marker LAMP-1 (Chen *et al.* 1985). CV-1 cells were infected with BPV virions for 30 min at 37°. (A) immunofluorescent detection of for BPV L1; (B) same cell stained for the lysosomic marker LAMP-1.

FIG. 6. Inhibition of BPV uptake by cytochalasin B. CV-1 cells were infected with either BPV (A, C, E) or SV40 (B, D, F) and viral antigen was detected by indirect immunofluorescence using an anti-BPV L1 monoclonal antibody and a polyclonal antiserum raised against denatured SV40 virions, respectively. (A) Pretreatment of the cells with cytochalasin B interferes with uptake of BPV virions (note staining on the cytoplasmic membrane) but allows penetration of SV40 into the cytoplasm (B). Pretreatment with taxol inhibits the uptake of neither BPV (C) nor SV40 virions into the cytoplasm (D), although a retardation of nuclear translocation compared to the untreated cells could be observed (E and F).

2E and 2F). However, by electron microscopy no particle was detected bound to the nuclear membrane although occasionally single virions or virus containing vesicles were located close to the nuclear membrane. This suggests that disassembly and release of the virions from the vesicles take place in the cytoplasm and no intact virus reaches the nucleus or the nuclear membrane. By immunofluorescence, the L1-specific signals were still found to be overlapping with the L2 protein (Figs. 2E and 2F). Practically all fluorescent signals had disappeared from the cytoplasmic membrane, indicating that the uptake process was completed.

After 90 min

Almost all cells showed a predominant nuclear staining pattern (Figs. 2G and 2H). The cytoplasm was cleared from most signals and the vast majority of the BPV capsid antigen was located in clusters in the nucleus. However, despite of the intensity of the signals obtained by immunofluorescence, no virions could be found by electron microscopy.

Previously a neutralizing monoclonal antibody 5B6 (Roden *et al.,* 1994b) to the BPV1 L1 was described which does not interfere with binding of virions to cells. When BPV virions were incubated with a 1:100 dilution of this antibody before exposure to the cells, we observed nuclear staining of the BPV L1 protein, suggesting that the neutralizing antibody could not inhibit BPV binding, uptake, or nuclear transport.

After overnight incubation

The fate of incoming virions was examined by immunofluorescence. We were interested in examining the final fate of incoming viral capsid antigen in the infected cells. After primary exposure to the virions, the cells were incubated overnight and then fixed to detect remaining BPV antigen by immunofluorescence. In almost all cells the nucleus was cleared from any signals, and lumps of fluorescent label were located in the cytoplasm, which was highlighted by α-actinin staining (Fig. 3C). Counterstaining with Golgi marker protein (anti-golgi-β-COP; Allan and Kreis, 1986) revealed that the capsid proteins colocalized with the Golgi apparatus, suggesting that the incoming proteins were discharged by the Golgi complex (compare Figs. 3A and 3B).

BPV virions are transported in phagosomes

The invagination of the plasma membrane resulted in the formation of endocytic vesicles as shown in Figs. 4C and 4D. Most vesicles were smooth, rather than coated, vesicles, and almost all virions inside were "full," DNA-containing virions. To find out whether some of the BPV-containing vesicles in the cytoplasm were late phagosomes, derived from fusion of endocytic vesicles with lysosomes, double staining for BPV L1 protein and lysosome marker was performed (LAMP-1; Chen *et al.,* 1985). The overall staining pattern for both proteins was very similar and the big lumps, representing large vesicles, were virtually overlapping (Fig. 5). These results confirmed the identity of a part of the BPV-containing vesicles as late phagosomes.

Effect of chemicals on penetration and intracellular relocation of BPV virions

As shown above, BPV virions bind to CV-1 cells, and the bound virions and capsid proteins can be rapidly transported into the cytosol and the nucleus (Fig. 2). To further characterize the mechanisms involved in the early events of BPV infection, we used inhibitors of various cellular functions to interfere with the penetration and transport of BPV virions. As CV-1 cells have been extensively used to study the SV40 entry pathway (Upcroft, 1987; Shimura *et al.,* 1987), we used the same cells for BPV uptake and transport studies. The results are summarized in Table 1. The use of lysosomotropic agents has shown that prelysosomal vacuoles or lysosomes were involved in the uptake of various enveloped viruses (Superti *et al.,* 1987). We tested whether these compounds influence the entry of PV into CV-1 cells. NH_4Cl (20 m*M*), chloroquine (100 μM), monensin (10 μM), and butylamine (1 m*M*) were applied to raise the pH of the lysosomes. The results show that all lysosomotropic agents at doses inhibiting the infection of SV40 (Upcroft, 1987; Shimura *et al.,* 1987) or other viruses (Helenius *et al.,* 1980) did not inhibit BPV transportation, as judged by immunofluorescence.

When microfilament-destroying cytochalasin B (5 μM) was used to treat CV-1 cells, the internalization was blocked completely (Fig. 6A). The virions remained on the plasma membrane even after 5 hr incubation at 37°. Interestingly, SV40 seemed to use different mechanisms in penetrating through the plasma membrane since treatment with the same dose of cytochalasin B had little effect on the uptake of SV40 (Fig. 6B), although the transport of the virions to the nucleus was slower than in untreated cells.

Microtubule-affecting agents were examined for the inhibition of BPV transportation into CV-1 cells. Cells were pretreated for 30 min at 37° with Vinblastine (1 μM), podophyllotoxin (10 μM), colcemid (0.7 μM), colchecine (10 μM), and taxol (10 μM) before BPV virions were added to the medium. Entry and transportation of the virions were determined by immunofluorescence. Under the given experimental conditions, only the treatment with taxol inhibited the transport of BPV, as well as SV40, from the cytoplasm into the nucleus (Figs. 6C and 6D).

Uptake of virus-like particles

We were interested in determining whether synthetic virus-like particles (VLPs) behave like complete infec-

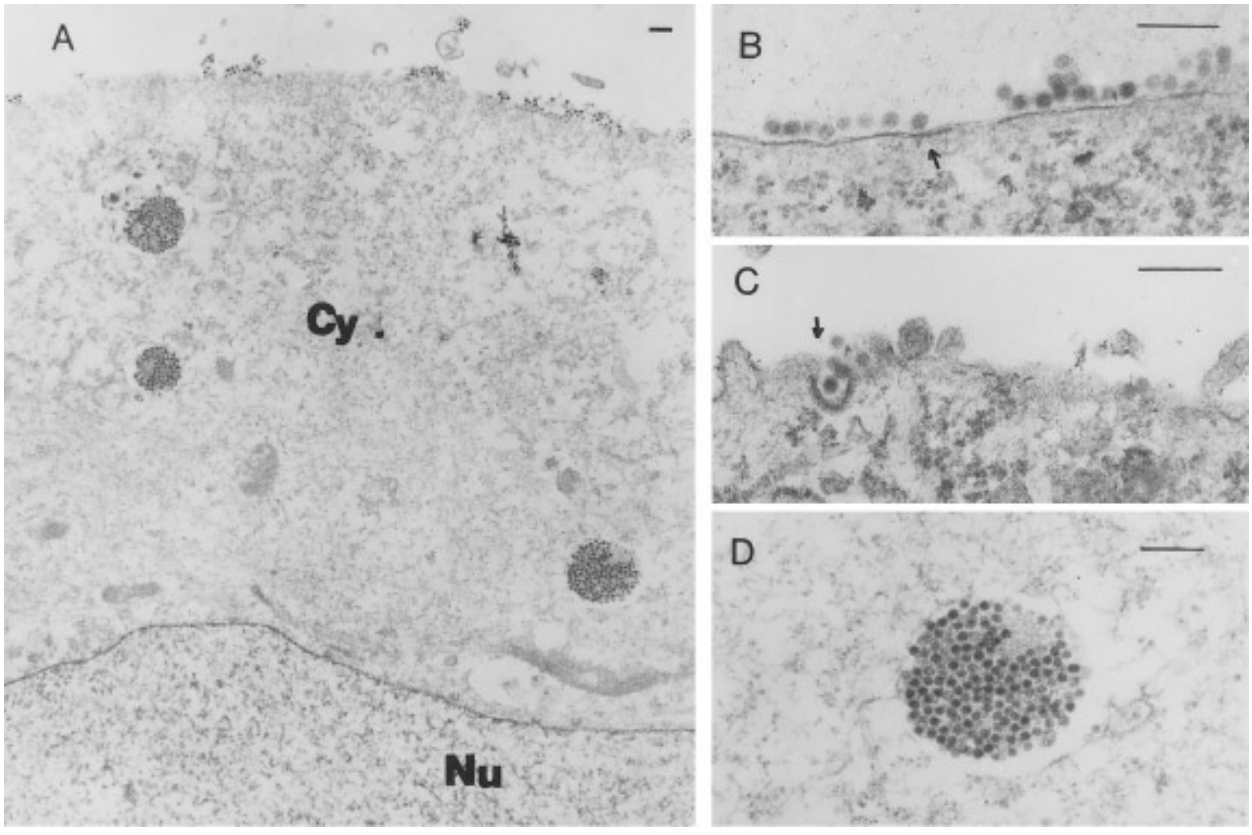


FIG. 4. Binding, uptake, and cytoplasmic transport of BPV virions analyzed by electron microscopy. CV-1 cells infected with BPV virions were incubated at 4° for 1 hr (B) and then shifted to 37° for 30 min (A, C, and D) and electron microscopically analyzed for subcellular localization of virions as described under Materials and Methods. (A) Overview to demonstrate the localization of BPV virions on the cytoplasmic membrane and in endocytotic vesicles in the cytoplasm (Cy) in various distances to the nucleus (Nu). (B) Membrane-bound BPV virions. The area depressed by the binding of virions is marked by an arrowhead; (C) particle being envaginiated (arrowhead); (D) shows a blow up of one of the cytoplasmic endosomes from (A) which contains more than 100 virions in this section. The size bars represent 250 nm.

tious BPV virions in the uptake test. We purified HPV11 VLPs assembled from the L1 protein either alone or together with the L2 protein from baculovirus-infected insect cells. After exposure to CV-1 or C127 cells the proteins were detected by immunofluorescence as shown for the BPV virions (data not shown). No differences were seen between L1 and L1/L2 particles. The process and time course of uptake were similar to those observed for complete BPV virions, indicating that the L2 protein plays no crucial role in the uptake or transport of the viral particles.

DISCUSSION

To start a productive infection, papillomaviruses must be able to bind to a putative receptor on the surface of their target cells and then be transferred across the plasma membrane into the cytosol. The viral DNA has to be transported to the nucleus for gene transcription and DNA replication. So far, it is unknown whether the disintegration of the virions takes place in the nucleus as it was described for mouse polyoma virus and SV40 (Mackay and Consigli, 1976; Barbanti-Brodano *et al.,* 1970; Clever *et al.,* 1991).

In this study we have analyzed the time course of the above events, when CV-1 or C127 cells were exposed to native BPV virions or HPV11 VLPs. We were able to inhibit the transport process specifically by cytochalasin B and taxol. We observed that after binding to the cells, the whole process of internalization and transport of the vast majority of virions into the nucleus, as assessed by the detection of the major capsid antigens using immunofluorescence, could be completed as fast as 90 min. It is assumed that some virions are being transported at an even higher rate. To this time, a strict colocalization of the L1 and L2 signals was evident. These results seem to be somewhat contradictory to a recent report (Christensen *et al.,* 1995), where the neutralization of infection by BPV virions using an anti-capsid antibody could be achieved even 8 hr postinfection. This result was interpreted as the virions remaining on the cell surface for a long time and, thus, accessible for the antibodies. At least it has to be assumed that uncoating did not occur in that time span. One explanation for these discrepancies between our study and the report by Christensen *et al.* could be the observation that in some of our experiments the uptake of the virions was completely delayed when

TABLE 1

Effects of Chemicals on the Papillomavirus Entry

Chemicals	Conc.	Distribution (30 min)	Distribution (60 min)	Distribution (90 min)
Control (non)	N/A	Cytoplasmic	Nuclear–membrane	Nuclear
Lysosomotropic reagents				
Chloroquine	100 μM	Cytoplasmic	Cytoplasmic	Nuclear
Butylamine	1 m*M*	Cytoplasmic	Cytoplasmic	Nuclear
Monensin	10 μM	Cytoplasmic	Cytoplasmic	Nuclear
NH_4Cl	15 m*M*	Cytoplasmic	Cytoplasmic	Nuclear
Amantadine	1 m*M*	Toxic to cell	ND	ND
Microtubule-disruping chemicals				
Colcemid	0.7 μM	Cytoplasmic	Cytoplasmic	Nuclear
Colchicine	10 μM	Cytoplasmic	Cytoplasmic	Nuclear
Podophylloxin	10 μM	Cytoplasmic	Cytoplasmic	Nuclear
Taxol	10 μM	Cytoplasmic	Cytoplasmic	Nuclear membrane
Vinblastine	1 μM	Cytoplasmic	Cytoplasmic	Nuclear
Microfilament-disrupting reagent				
Cytochalasin B	5 μM	Membrane	Membrane	Membrane

Note. The localization of BPV virions was determined by immunofluorescence. The cells were preincubated with the above compounds as described under Materials and Methods. BPV virions were added for 1 hr at 4°. The cells were then washed and the temperature was shifted to 37°.

the cells were used at a high density. However, we cannot exclude that the massive transport of virions through the cell would have lead to an abortive infection. In this case only a slight and not readily detectable portion of the viruses could have entered the cells in a delayed fashion to establish a successful infection. Although not directly addressed, a recent study (Volpers *et al.,* 1995) suggested that 60 min at 37° were sufficient to allow internalization of HPV33 VLPs.

As judged from the intensity of the signals obtained in immunofluorescence, a huge amount of virions were bound per cell and then transported toward the nucleus, confirming the report by (Volpers *et al.,* 1995) where a high number of receptor molecules for HPV33 was detected (2×10^4 per HeLa cell). Surprisingly, this artificially high number of virions did not seem to lead to an overload or saturation of the transport mechanisms since the whole process of transport to the nucleus was completed in as little as 90 min. These findings are very similar to the transport of polyoma virions where it was reported earlier (Mackay and Consigli, 1976) that the majority of virions are located in the cytoplasm after about 1 hr and some virions reach the nucleus in as little as 15 min.

No differences between empty virus-like particles, either consisting of L1/L2 or the L1 protein alone, and native BPV virions were observed when analyzed for their internalization and transport using immunofluorescence. This indicates that the virus-like particles bear all required structures involved in the binding and transport processes.

It was reported earlier that although so far all antibodies known to inhibit binding to the cell surface were specific for the L1 protein (Christensen and Kreider, 1990), antibodies against the L2 protein can neutralize infection of BPV virions by a yet unknown mechanism. When we preincubated BPV virions with such an anti-BPV L1 antibody 5B6 (Roden *et al.,* 1994b) before exposing cells to the virions, we observed the transport of the L1/L2 complexes together with the antibody into the nucleus. Therefore, inhibition of internalization or targeting the nucleus can be excluded. Since we have not detected complete virions in the nucleus it might be hypothesized that the antibody leads to premature release and degradation of the viral DNA before the nucleus is reached.

To allow detection of BPV virions or viral capsid antigens, we exposed the cells to artificial large amounts of virions. Full virions bound to be plasma membrane were readily detectable by electron microscopy. The virions were taken up by the cells by endocytosis and ended up in relatively large vesicles. Occasionally smaller vesicles were observed, containing only few virions. However, we believe that these vesicles are a result of the large number of virions the cells were exposed to and that they do not represent an abortive infection as reported for SV40 (Maul *et al.,* 1978; Hummeler *et al.,* 1995), since they were not clathrin-coated endosomes. For polyoma virus the existence of two different transport pathways was reported (Mackay and Consigli, 1976). While infectious virions were found in monopinocytotic vesicles, empty

capsids accumulated in large phagosomic-like vesicles. However, the latter ones were not able to support the transport of labeled capsids or capsid proteins into the nucleus as we have shown to be the case for transport of papillomavirus virions. Furthermore, in contrast to the findings of Mackay and Consigli (Mackay and Consigli, 1976) for polyomavirus, we assume that the large cytoplasmic vesicles contain mostly DNA positive virions since (1) electronmicroscopy revealed full particles and (2) our virus preparation contained only minor amounts of empty virions. Immunofluorescence counterstaining with an antibody specific for a lysosomal component revealed that the majority, but not all, of virus containing endosomes were fused with lysosomes. Despite the frequency of virions in the cytoplasm and the strong immunofluorescence signals found on the nuclear membrane or in the nucleus we were not able to observe any complete particles in the nucleus or near the nuclear membrane. Therefore, it has to be concluded that disintegration of the virions occurs in the cytosol, presumably under the influence of lysosomic factors. The uncoated, and presumably disassembled, capsid components might then migrate to the nucleus via their nuclear localization signals present on the L1, as well as the L2, proteins (Zhou *et al.,* 1991; Sun *et al.,* 1995).

It was reported by Volpers *et al.* that a capping structure of viral antigen as a result of multivalent receptor binding occurred on HeLa cells when exposed to HPV33 virus-like particles (Volpers *et al.,* 1995). No such structure was found on CV-1 or C127 cells, where the distribution of viral antigen was very homogeneous over the whole plasma membrane. However, using a cell line which was negative for internalization of virions bound virions condensed in a typical cap when cells were incubated at 37° (manuscript in preparation). Obviously the condensation of virus/receptor complexes occurred only at the higher temperature but could not evolve on CV-1 or C127 cells because of their highly efficient internalization machinery. The most likely explanation for the observed capping of internalization-capable HeLa cells is the presence of an anti-capsid antibody in the experiments described by Volpers *et al.,* before the cells were shifted to 37°. The presence of this antibody most likely delayed internalization, allowing the formation of a receptor cap.

Since we did not observe entire BPV virions being transported into the nucleus, we cannot rule out that the PV uncoating process takes place in the cytosol compartments. Both polyomavirus and SV40, which share similarity in viral structure, enter the nucleus for uncoating (Clever *et al.,* 1991; Mackay and Consigli, 1976; Barbanti-Brodano *et al.,* 1970). However, the uncoating process seems to be very fast as both polyomavirus and SV40 never accumulate in the nucleus (Mackay and Consigli, 1976; Maul *et al.,* 1978).

Lysosomotropic reagents such as chloroquine, monensin, NH_4Cl, and colcemid did not interfere with BPV penetration and nuclear transportation. It was reported that infection by some types of enveloped RNA viruses, such as vesicular stomatitis virus, Semliki Forest virus, Sindbis virus, and influenza virus, which all propagate in the cytoplasm, is inhibited by lysosomotropic agents. Therefore, in the process of infection with these viruses, lysosomes probably play a vital role (Helenius *et al.,* 1980; Coombs *et al.,* 1981; Yoshimura *et al.,* 1982; Cassell *et al.,* 1984). However, although many BPV virions accumulated in lysosomes as revealed by EM, a successful infection may not utilize lysosomes to establish infection.

The cytoskeletons, which consist of microtubules, microfilaments, and intermediate filaments, are believed to play a role in cell shape, movement, and division as well as in endocytosis. We showed that cytochalasin B affects the receptor-mediated endocytosis of BPV virions. Cytochalasin B destroys microfilaments and their function (Wibo and Poole, 1974), indicating that the microfilaments play an important role in PV penetration. The uptake of SV40 was not inhibited as revealed by our study and by others (Shimura *et al.,* 1987), suggesting that PVs and SV40 may use different receptors and different entry pathways in the early phase of infection despite the similar structures in both viruses. The immunofluorescence showed that the microtubule-disrupting agent taxol could inhibit both BPV and SV40 transport to the nuclei of infected cells. The inhibition was taking place after BPV and SV40 were internalized. Such inhibition, therefore, affects the endosome movement because such vesicles are fixed to or move along microtubules in the cytoplasm (Bhisey and Freed, 1971; Collot *et al.,* 1984). Although many other microtubule-affecting chemicals (vinblastine, podophyllotoxin) inhibit SV40 infection at the same concentration as we used in this study (Shimura *et al.,* 1987), we did not find such inhibition for both BPV and SV40. Such discrepancies could have resulted from different cells used for the assays. However, we also cannot exclude the possibility that taxol inhibits the transport of BPV virions due to an as yet unknown activity, which does not involve microtubules. We were not able to significantly inhibit focus formation by pretreatment of cells with either cytochalasin B or taxol (data not shown). However, because of toxic effects, both chemicals can only be applied to the cells for a brief period of time and bound BPV virions might have been taken up by the cells after removing the chemicals. Therefore, it remains unclear whether both agents have the proposed functional inhibition on infections by papillomaviruses.

When the process of transport was followed for a prolonged time (overnight) we observed deposition of the capsid antigens in the Golgi apparatus. While this result is likely of less importance for the viral life cycle, in which the cells are exposed to a much lower virus load during naturally occurring infection, it might have some implications when virus-like particles become available for vaccination in the future. Locally high concentrations of parti-

cles might lead to uptake and finally processing of capsid antigen followed by presentation through MHC class I molecules, possibly leading to a cytotoxic T-cell response.

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